THE EFFECT OF THE MUTAGEN ACRIDINE ORANGE ON THE DNA OF THE MINUTE PLANKTON CRUSTACEANS, *DAPHNIA*

by

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Presented to the Faculty of the Honors College of
The University of Texas at Arlington in Partial Fulfillment
of the Requirements
for the Degree of

HONORS BACHELOR OF SCIENCE IN BIOLOGY

THE UNIVERSITY OF TEXAS AT ARLINGTON

May 2022

ACKNOWLEDGMENTS

I would like to acknowledge Dr. Sen Xu for being my mentor on this research project and for allowing me to conduct research in his genetics lab. I would also like to acknowledge Marelize Snyman for helping my peer, Eesha Khan, me during this project from early in the summer until now. Furthermore, I would like to thank Eesha Khan, my research lab partner, for the work we did together in the lab over the summer and fall and for our ride through college together.

I would like to thank my family, especially my mom, for supporting me over the years for aiding me to where I am today. Without their support, I would not be able to accomplish what I have in my life. Lastly, I would like to thank my cat Sam and my old cat Dean for providing me support when I wrote my thesis.

November 10, 2021

ABSTRACT

THE EFFECT OF THE MUTAGEN ACRIDINE ORANGE
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The University of Texas at Arlington, 2022

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Acridine Orange is a fluorescent dye, which can smoothly permeate a cell membrane. Acridines are considered to produce frameshift mutations, where the insertion or deletion of a base pair occurs. *Daphnia*, plankton crustaceans, were used as the organism to interact with the mutagen as they have a prompt life cycle and can be cultivated in large populations. After isolating the *Daphnia*, they were subjected to different treatments of Acridine Orange to indicate which one the *Daphnia* can survive in, but not thrive. The determined mutagen concentration used for the *Daphnia* is 1.5 μg/mL. The sequenced F1 generation indicated the presence of indels, and the calculated mutation rate indicated a higher rate for the *Daphnia* clones than the control. Further research is being done to specify the type of mutation and the mutations of the babies. In future research, it can be determined that for the babies, it will likely be a frameshift mutation.

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CHAPTER 1

INTRODUCTION

Inducing mutations in a genome has provided information about multiple genes and what they do or do not control. To begin this process, mutagens are employed to prove whether they can carry out a mutation within an organism's genome. To understand which genes cause a specific phenotype in an animal, forward genetic screening is utilized. Forward genetic screening can be considered as a way to indicate where specific mutations occur and their implications.

1.1 Inducing Mutations

When inducing mutations, there are typically three methods used, which include chemical, radiation, and transposon insertion. Of the three, the method referred to as chemical is completed by inducing a mutation through a chemical mutagen. The two categories of chemical agents that work with Deoxyribonucleic acid (DNA) include nucleoside analogs and intercalating agents. During replication, nucleoside analogs can be added into DNA, and it may be noted that they have a comparable structure to regular nucleotide bases. Since nucleoside analogs have different base-pairing rules than those of which they take the place, they are allowed to induce mutations. The other category, which is intercalating agents, alters the DNA molecule by going through the nitrogenous bases in a stack formation. This results in an alteration of the arrangement of the base pairs in terms of the spacing between them. This influences DNA polymerase during DNA replication in that when DNA polymerase is replicating nucleotide base pairs, it may insert an extra

nucleotide, resulting in an insertion, or it may skip the replication of nucleotides, resulting in a deletion. Both these processes are called frameshift mutations, which is a common mutation resulting from chemical mutagens (Parker et al. 2016). In frameshift mutations, there is an occurrence of indels. Indels are considered the insertions and deletions aspect when it is less than one thousand base pairs. The reason it is considered a frameshift mutation is that if the indel is in the coding region of the DNA, the amount that was deleted or inserted can be divided by three. The name frameshift mutation also sparks from the point of the codon being changed for the remaining nucleotide base pairs. During the process of sequencing, if the indels have more than or equal to forty base pairs, then the technology used for sequencing is able to recognize the indels. If the indels have more than 40 base pairs, then it is more difficult to recognize the indels (Sanders et al. 2016).

1.2 Origin of Mutations

1.2.1 Spontaneous Mutations

Mutations can arise from being spontaneous to being induced. Spontaneous mutations arise when there is a mishap in DNA replication (Parker et al. 2016). Spontaneous mutations are not the typical mutations to screen for when completing forward genetic screening as the mutation rate has been found to be considerably low (Gondo et al. 2017). The mistake rate was found for spontaneous mutations by DNA polymerase in a billion base pairs that were replicated to be only one (Parker et al. 2016). In a study Gondo referenced that was done on mice, the mutation rate was found the magnitude to be around three orders decreased in comparison to the induced mutagen. It is noted how screening for these spontaneous mutations often takes much work and time, but

if this were to be completed it may be used widely as a source for others (Gondo et al. 2017).

1.2.2 Induced Mutations

Induced mutations arise when mutagens are exposed to the organism, including chemical mutagens. The mutation rate can be altered to increase more than 1000-fold (Parker et al. 2016). Therefore, induced mutations are much easier to screen for as there is much more chance for the presence of indels to be identified.

1.3 Mutagens

1.3.1 Typical Mutagen Characteristics

Mutagens used to induce mutations are typically considered carcinogens, which are substances with the ability to cause cancer. Some typical carcinogens include intercalating agents such as polycyclic aromatic hydrocarbons, which is a type of combustion product. In lab settings, both acridine orange and ethidium bromide are often used to view DNA through staining it. Both these substances are intercalating agents and possible mutagens (Parker et al. 2016).

1.3.2 Past Mutagen Experiments on Daphnia

Past experiments have been performed to induce mutations on the model organism *Daphnia*. In a study done by Snyman, the mutagen ethyl methanesulfonate was introduced to *Daphnia* and for three species, the result for the 10 mM concentration was a mean rate of 1.17×10^{-6} for base substitutions. Additionally, the result for the 25 mM indicated an increase in the rate of mutation with a total of 1.75×10^{-6} for base substitutions (Snyman et al. 2020). In another study done by Keith, the mutagen Cadmium was introduced to *Daphnia*, which resulted in an increase in adenine:thymine to guanine:cytosine base-pair

rates of mutation. However, there was a decrease in the rate of mutation for cytosine:guanine to guanine:cytosine. The average mutation rate induced by Cadmium in the *Daphnia* was shown to be 1.69 x 10^{-9} (Keith et al. 2021).

1.4 Acridine Orange

1.4.1 Acridine Orange Description

Acridine orange (AO) is noted as a fluorescent dye typically used to stain living cells and can smoothly permeate a cell membrane. It can typically be found to collect in low pH environments, lysosomes, because AO is a weak base. Lysosome membranes contain a proton pump reliant on ATP. As the lysosome protonates AO, it is stuck there (Vermes and Haanen 1994). Through the recognition that AO mutations cannot be returned through mutations of base substitutions and how mutations caused by acridines halt the creation of proteins, Brenner suggested that mutations caused by acridines would result in the insertion or deletion of a base pair, indicating acridines are mutagens with the ability to create frameshift mutations (Reha-Krantz 2013).

1.4.2 Concentrations, Mutation Rate

In lab settings where AO is used for a staining method, the concentration used is typical to be of 5 x 10^{-6} M. The pH of the substance is also typical to be neutral with the range of 7.2-7.4. A high concentration of AO would be noted as 10^{-2} M to 10^{-3} M as in this concentration, the cell has been known to be severely altered or be terminated (Zelenin 1999). A previous experiment on Escherichia coli, a bacteria, had been done but nothing with AO's effect on *Daphnia* has been done. The tested concentration on Escherichia coli was 2 x 10^{-6} M, which indicated a 15-fold rise in the rate of mutations for the wild-type strain of the bacteria (Hass and Webb 1979). In another experiment regarding Escherichia

coli in the dark, the polymerase I-deficient strain of the bacteria indicated an increase in the rate of mutation by 14-fold from 2 μM of AO being induced (Webb and Hass 1984).

1.5 Daphnia

1.5.1 Daphnia as the Model Organism

Daphnia are used as the model organism in various lab settings for numerous amounts of factors. They are minute crustacean organisms and are keystone species present in majority of freshwater ecosystems. *Daphnia* are considered to encounter stress factors created by both man-made and natural means (Altshuler et al. 2011). This is important as they can adapt to conditions very quickly and have a high environmental tolerance (El-feky et al. 2020). *Daphnia* have had their whole genome sequenced, allowing for screening to be done through mapping the experimental genome to the control genome. Their life history and ecology are very well studied, allowing experimenters to understand their patterns. In mutation-related experiments, *Daphnia* are used to understand how stress factors affect rates of mutation (Altshuler et al. 2011). *Daphnia* can be cultivated in large populations, allowing this to be a great organism for use in a lab for the case of multiple trials. Its prompt life cycle allows for *Daphnia* to be cultivated quickly as well, which is important for a lab setting because organisms will need to be constantly cultivated to perform the experiments (El-feky et al. 2020).

1.5.2 Asexual vs Sexual Reproduction

In general, *Daphnia* use the reproduction method of cyclical parthenogenesis, which involves sexual and asexual reproduction. Sexual reproduction is induced when environmental stress factors take place, including an alteration in the photoperiod or presence of predators (Altshuler et al. 2011). Sexual reproduction involves the females

creating haploid sexual eggs, which need to be fertilized through breeding with a male partner. In a shell, which is called the ephippium, the fertilized diploid egg rests till prepared to be delivered. Asexual reproduction is induced when conditions are favorable and factors such as a low presence of predators and ample food availability are present. Asexual reproduction involves adult female *Daphnia* creating clones of themselves by themselves that may be female or male (La et al. 2014). With this reproduction method utilized in the lab, the various lines of *Daphnia* can be noted and whether the mutation resulting from stress factors occur from a genetic standpoint (Altshuler et al. 2011).

1.6 Hypothesis

Based on the research completed on the mutagen acridine orange and the model organism *Daphnia*, the question to be researched is how the minute crustacean will be affected by acridine orange in terms of the adequate concentration and the presence of indels, indicating frameshift mutations. It is hypothesized that the concentration to be determined will need to be strong enough to induce the mutation, but not to where the organism will be terminated. The ample concentration is hypothesized to be around the fifty/fifty mark where there might be a slightly higher death rate for the specific concentration. This range may be within 2×10^{-6} M AO and 5×10^{-6} M AO. It is also hypothesized that there will be the presence of indels in the genome of the sequenced F1 generation of the *Daphnia* compared to the control genome.

CHAPTER 2

METHODOLOGY

2.1 Examined Organisms

For this experimental study, cyclically parthenogenetic *Daphnia pulicaria* isolates (AroMoose, Pine 1, 3L2-1, and Warner 5) in a series of five *Daphnia* per trial for two trials each were utilized. The mentioned animals were maintained in a lab setting in lake water that was artificially created, which is referred to as *Daphnia* combo. The light conditions for the animals were a period of 16 hours of light and 8 hours of dark. The temperature the animals resided at was 18 °C. These animals were originally obtained in the United States and Canada from different lake communities. For food, the animals were treated two times a week with *Scenedesmus obliquus*, green algae (Snyman et al. 2020).

2.2 Identifying Adequate Acridine Orange Concentrations

Daphnia were tested through six different concentrations to determine the endurable concentration to be used for the remainder of the experiment. The concentrations were 0.75 µg/mL, 1 µg/mL, 1.25 µg/mL, 1.5 µg/mL, 1.75 µg/mL, and 2 µg/mL. These concentration ranges were acquired from conducting numerous trials. Since a past experiment utilizing the effect of AO on *Daphnia* has not been done yet, a reference to similar experiments in which AO was implemented was noted in past research (Snyman et al. 2020).

These concentrations were put on trial with females who reached maturation from the AroMoose, Pine 1, 3L2-1, and Warner 5 *Daphnia pulicaria* isolates. For each

concentration, two trials were performed with each trial containing 5 isolates at a time. Five *Daphnia* of each isolate were isolated and placed in plastic vial with the following concentrations of 0.75 µg/mL, 1 µg/mL, 1.25 µg/mL, 1.5 µg/mL, 1.75 µg/mL, and 2 µg/mL. The *Daphnia* were allowed to sit with exposure to the mutagen for a total of four hours with monitoring. These organisms were relocated to the artificial lake water, known as *Daphnia* Combo. To determine the survival rate for each concentration, analysis was done 24 hours following the experiment (Snyman et al. 2020).

2.2.1 Determined Concentration

From the concentration portion of the experiment, the determined concentration of 1.5 µg/mL was utilized for the remainder of the experiment. The F0 generation of these organisms was allowed to reproduce to create plenty of the F1 generation.

2.3 DNA Extraction

2.3.1 Sequencing

DNA was extracted from the F1 generation of the organisms that experienced the 1.5 µg/mL mutagen concentration. The organisms used for extraction were the 3L2-1 and Warner 5 isolates, which also each produced mutant lines referred to as the dark purple clone, light blue clone, and silver clone for the 3L2-1 isolates. The mutant lines for Warner 5 were referred to as the dark purple clone, light blue clone, and red clone. The extraction technique used was the Cetyl Trimethyl Ammonium Bromide Method, otherwise known as CTAB. CTAB involves a homogenization buffer being pre-warmed and applying this to a microcentrifuge tube that has 50-150 *Daphnia*. This is then ground with blue pestle, to which the pestle is rinsed with additional homogenization buffer to the tube. Incubation is done for an hour at 65°C, then centrifugation is done. Then the substance is relocated to

new tubes and Chloroform:Isoamyl alcohol is introduced and centrifuged again. RNase is added to the upper later and allowed to incubate. Transfer to a new tube was completed and allowed to mix with cold ethanol. Incubation and centrifugation were completed. H2O was added, and the sample was allowed to sit overnight to re-dissolve. Utilizing a Thermo Fisher Qubit 4.0 Fluorometer, the concentrations of the samples of DNA were quantified. The quality of the DNA was examined on a 2% agarose gel through electrophoresis. DNA was sequenced on an Illumina platform with 150-bp paired-end reads (Snyman et al. 2020).

2.3.2 Analysis of DNA Genome

The genome that resulted from sequencing was mapped to the *Daphnia pulicaria* reference genome utilizing the Burrows-Wheeler Alignment Tool BWA-MEM function. The function MarkDuplicates from Picard tools indicated the PCR duplicates. The function SAMtools eliminated reads that mapped to multiple locations. This process decreases the chance of false positives.

Indels were called utilizing the BCFtools call function. These were then analyzed through the BCFtools-stat function (Snyman et al. 2020). The BCFtools-stat function compared samples with each other to recognize indels. The function ensured that the indels were unique and not shared between the remaining mutant lines. The isolates that had the best sequencing results remained in the analysis process, while the isolates with inadequate results were removed from the experimental data.

2.4 Analysis through Graphs and Calculated Mutation Rate

A line graph was utilized to create a survival rate graph from the concentration trials. Bar Graphs were created to indicate the indel amount for the different isolates and compare the number of indels for the control isolate compared to the 3L2-1 and Warner 5

isolates. To calculate the mutation rate, the total number of indels in the control genome was subtracted from the total number of indels in the genomes of the isolates. This value was then divided by 200,000,000, which is the total number of base pairs that *Daphnia* have. This resulting value would indicate the mutation rate.

CHAPTER 3

RESULTS

3.1 Determined Concentration Utilized in Experiment

During the first portion of the experiment, multiple trials were run to determine the ample concentration for use through the inducing mutations portion of the experiment. The lowest concentration of 0.75 µg/mL resulted in the highest survival percentage with almost all isolates surviving, except the AroMoose isolate. Both the 1 µg/mL and 1.25 µg/mL concentrations experience similar survivorship rates, except for the AroMoose isolate, in which the survivorship for AroMoose drastically drops with the 1.25 µg/mL concentration. In the 1.5 µg/mL concentration, middle values can be found for the exact survival percentages, with the AroMoose isolate being on a separate scale compared to the other isolates. A trend seen within the *Daphnia* isolates is that for all of them, the concentration of 2 µg/mL resulted in a zero-survival rate, indicating this concentration is lethal (Table 1). The wanted fifty-fifty mark with a slightly higher death rate can be noted at the 1.5 µg/mL line. The 3L2-1 Clones and Warner 5 Clones were represented and indicated 1.5 µg/mL would be the adequate concentration for the mutagen to be induced, but not terminate the organism (Graph 1).

Table 3.1: The exact survival percentages of the *Daphnia* isolates resulting from the mutagen, acridine orange

Acridine Orange Concentration (µg/mL)	Exact Survival Percentages (%)			
	AroMoose	Pine 1	3L2-1	Warner 5
0.75	96	100	100	100
1	56	93.33	93.33	73.33
1.25	8	93.33	86.67	80
1.5	4	80	86.67	60
1.75	0	66.67	46.67	20
2	0	0	0	0

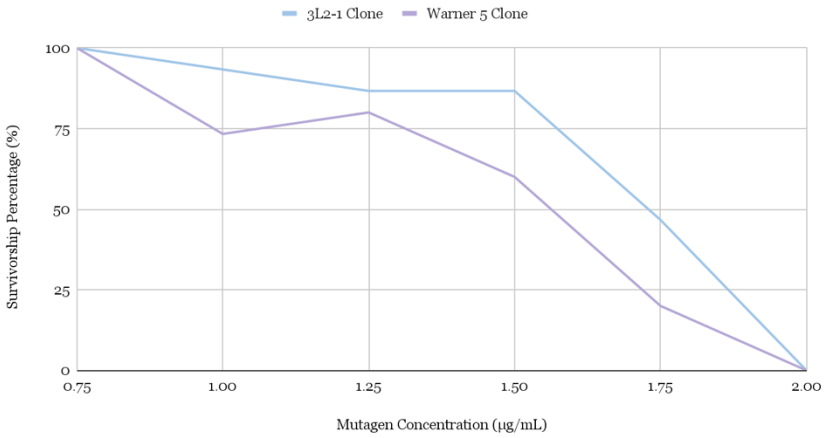

Figure 3.1: The visual representation of the effect of acridine orange on the survivorship percentage of *Daphnia*

3.2 Indels Present in *Daphnia* Isolates

After the isolates were sequenced and BCFtools were used to call for indels, only the isolates that remained with the best results were used for the remainder of the experiment. These isolates that remained were the 3L2-1 isolate and the Warner 5 isolate,

while the AroMoose and Pine-1 isolate were disregarded in terms of indels and mutation rate.

3.2.1 3L2-1 Daphnia Clone Indels

The indels present in the different 3L2-1 *Daphnia* clones varied for each, with majority of the clones presenting with more indels than the control. The specific clones with more indels than the control were the Dark Purple Clone and the Silver Clone, which had the highest amount of indels overall. However, the Light Blue Clone presented with fewer indels between the clones and even had fewer indels when compared to the control genome. This may be attributed to an error (Figure 2).

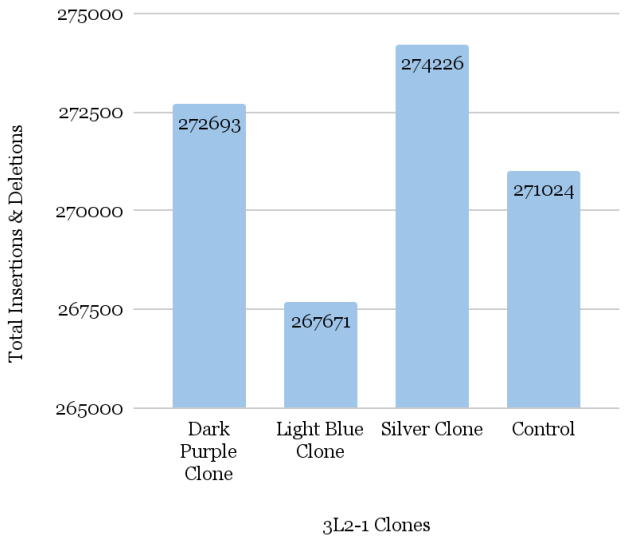

Figure 3.2: The total insertions and deletions (indels) for the 3L2-1 *Daphnia* Clones

3.2.2 Warner 5 Daphnia Clone Indels

For the Warner 5 clones, the indels indicated have little variance and are quite similar in value. The clones also presented with a higher amount of indels in comparison to the control genome. These clones are presented as the Dark Purple Clone, the Light Blue Clone, and the Red Clone. The Red Clone had the highest amount of indels in comparison

to the other clones, with an indel amount of 255440. The Light Blue Clone had fewer indels than the other clones with 250520 indels (Figure 3).

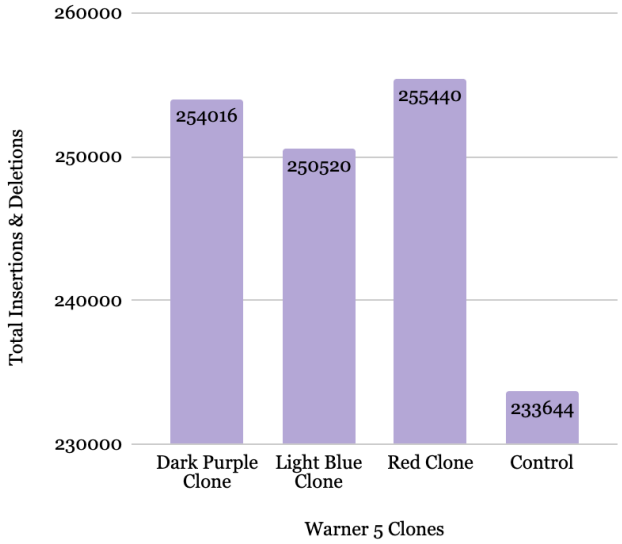

Figure 3.3: The total insertions and deletions (indels) for the Warner 5 *Daphnia* Clones

3.3 Calculated Mutation Rate

3.3.1 3L2-1 Clones Mutation Rate

After the mutation rate was calculated for the 3L2-1 clones, it can be noticed how the Silver Clone had the highest rate of mutations at 1.60 x 10-5, with the Dark Purple Clone second at a rate of 8.35 x 10-6. There was a negative value calculated for the Light Blue Clone. This may be attributed to an error (Table 2).

Table 3.2: The calculated mutation rate for the 3L2-1 Clones from dividing subtracting the control indel from the clone indel and dividing the value by the total base pairs in the reference *Daphnia* genome.

3L2-1 Clones	Mutation Rate
Dark Purple Clone	8.35×10^{-6}
Light Blue Clone	-1.68×10^{-5}
Silver Clone	1.60×10^{-5}

3.3.2 Warner 5 Clones Mutation Rate

The calculated mutation rate for the Warner 5 Clones presented with all positive values, meaning the rate of mutation for all the clones was higher than the control genome. The Dark Purple Clone had the highest rate of mutation at 1.02×10^{-4}, indicating a 10-fold rise in the rate of mutations. The Light Blue Clone had the lowest rate of mutation at 8.44×10^{-5} (Table 3).

Table 3.3: The calculated mutation rate for the Warner 5 Clones from dividing subtracting the control indel from the clone indel and dividing the value by the total base pairs in the reference *Daphnia* genome.

Warner 5 Clones	Mutation Rate
Dark Purple Clone	1.02×10^{-4}
Light Blue Clone	8.44×10^{-5}
Red Clone	1.09×10^{-4}

CHAPTER 4

CONCLUSION

4.1 Achieving Experiment Purpose

In this study, the effect of the mutagen acridine orange on *Daphnia* was experimented by determining the ample concentration that *Daphnia* would have the mutagen induced and the rate of mutation on the F1 generation's genome. It was shown how by utilizing a 1.5 μg/mL concentration level, that the mutagen would adequately be induced, to where the survival rate was towards the middle between 0% and 100%. This presents a concentration of acridine orange that can be utilized further. Mutation rate from the mutagen acridine orange on the genome of the F1 generation was also shown and can be utilized as a reference as well. These results can be compared to past research to show their validity and to show if any improvements can be made.

4.2 Analysis of Hypothesis

4.2.1 Analysis of Hypothesis for Determined Concentration

In the hypothesis, it was estimated that the determined concentration would be between or near the range of 2×10^{-6} M AO and 5×10^{-6} M AO. This portion of the hypothesis can be deemed supported as the determined concentration was 1.5 μg/mL AO, which results in 5.65×10^{-6} when converted. The base value of 5×10^{-6} M AO was researched from AO being used as a stain, so with the determined concentration being slightly higher than this stain value, it can be known that a higher concentration needs to

be used to induce the mutagen for indels to be present adequately when screening (Zelenin 1999). The base value of 2 x 10^{-6} M AO was researched from AO being used on Escherichia coli, but this value is a bit lower than the determined concentration, which could possibly mean that the bacteria does not need such a high concentration to have the mutagen induced in comparison to *Daphnia* (Hass and Webb 1979). After analyzing the survivorship graph, it can be supported how the concentration at the fifty-fifty mark was correctly chosen as the concentration of 1.5 μg/mL AO, was near this mark with slightly higher deaths than survivors.

4.2.2 Analysis of Hypothesis for Presence of Indels

The second portion of the hypothesis estimated that there would be an indication of indels after the F1 generation was sequenced and that the mutation rate would be higher for the *Daphnia* clones than the control genome. After analyzing the results, this portion can be supported as the 3L2-1 and Warner 5 clones both had the presence of indels, indicating the occurrence of a frameshift mutation. With the calculated mutation rates, it can be determined that there was a successful determination to see that there was an in fact increase in the rate of mutations resulting from acridine orange with comparison to the control *Daphnia* genome. The calculated mutation rates can also be compared to literature rates to determine accuracy. In an experiment with Escherichia coli, affected by a mutagen, there was a determined 15-fold increase in mutation rate, which can be compared to the 10-fold mutation rate resulting from acridine orange being inflicted upon the 3L2-1 clone (Hass and Webb 1979). In another experiment with the same bacteria, there was a 14-fold increase induced by AO, which can be compared to the 10-fold increase seen in this study's results (Webb and Hass 1984). These comparisons to research provide further support for

the hypothesis made of how indels would be present, indicating frameshift mutations. For one part of the results in the Light Blue Clone for the 3L2-1 Isolate, the negative value indicates that the rate of mutation in the mutant line was less than the control genome, which can be attributed to errors.

4.3 Errors Within Experiment

An error associated with the study would be when the indels were called and indicated a clone with fewer indels than the control. This error may be attributed to not adding enough of the mutagen in the concentration portion of the experiment. This means that an insufficient amount of the mutagen was prepared at the beginning of the experiment, leading to fewer indels being induced in the genome. Another reason for this could be how the specific clone has resistant properties. For this, the clone would be able to not be as susceptible to the mutagen and the mutagen would not affect the clone as much. In a study, it was found how there are some strains of *Daphnia pulicaria* have been found to resist toxic algae, which contains toxic metabolites (Clark et al. 2021). This research provides support for this reason for the possible error.

4.4 Future Directions

4.4.1 Research in Process in Xu Lab

This study is part of an ongoing experiment that will soon be to determine the phenotypic mutations present in the F2 generation of *Daphnia*. Ephippia, which are resting eggs, from the F1 generation is being dissected to remove the 1-2 eggs residing in the pouch. These eggs are then incubated for about 2-3 weeks, then placed under ultraviolet light. If no signs of development are present after checking each day for up to five days, then the eggs are removed from the ultraviolet light and placed in the incubator for about

a week. Signs of development are checked once again, but if there is none, the eggs are moved under natural light. Once the ephippia hatch, they are placed under natural light and fed with algae. The purpose of hatching these eggs will allow us to see those that have abnormalities and more specifically reproductive abnormalities. It is also important to note whether the babies of the F2 generation can asexually produce or not, or if they can produce ephippium or not. Some other characteristics researchers may be interested in would be if the babies swim abnormally or if they have additional limbs.

4.4.2 Future Experiments

Future experiments with similar purposes may also be researched. One research that would be with similar purpose is subjected *Daphnia* to ethyl ethane sulphonate (EES) or mustard gas, both of which are alkylating agents. EES is an agent similar to ethyl methane sulphonate, to which these two results can be compared to determine accuracy. These agents can cause mutations within DNA that can and cannot replicate. These mutagens may lead to transitions, insertions, deletions, or transversions in the genome (McClean 1999).

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BIOGRAPHICAL INFORMATION

Aleema Haq will graduate with an Honors Bachelor of Science in Biology from the University of Texas at Arlington. She holds leadership positions at the University of Texas at Arlington as the Public Relations officer of the Minority Association of Pre-Medical Students and the Videographer position in the Muslim Student Association. She has an interest in video making and editing and uses her camera to record videos of herself, family, and friends doing activities such as daily life or travel trips to keep for memories, or occasionally post on YouTube. She currently plans to apply to a medical assistant position to gain more knowledge and experience in the medical field. She also volunteers in Hospice and other clinical settings. In the future, Aleema plans to apply to medical school and carry out a career in medicine.